Investigation of Two Aneugenic Controls for CREST Micronucleus

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Figure 1. Representative Images of Clastogen and Aneugen Signals after CREST Staining

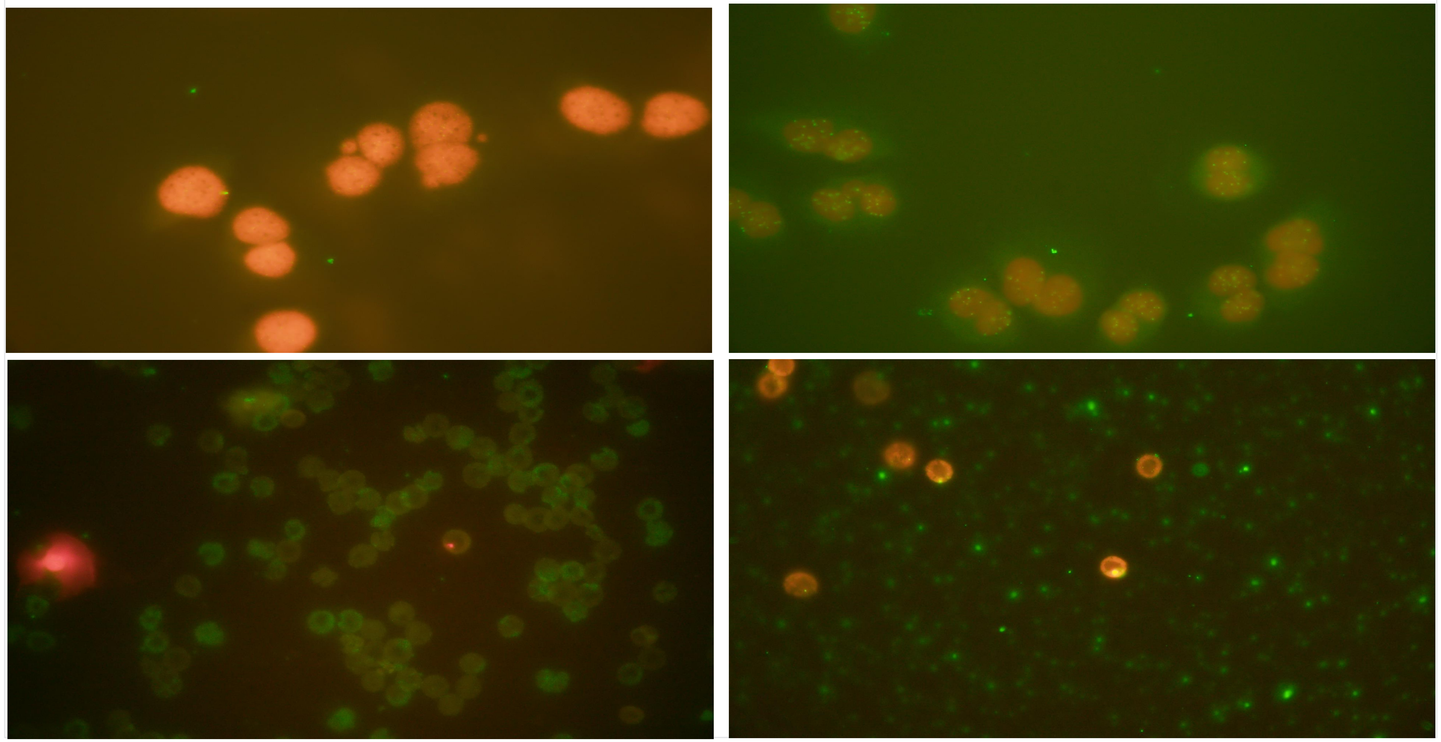

Figure 2. Individual Animal Micronucleus Data

Treatment	Animal No.	%PCE	MNPCE	PCE	%MNPCE
0.9% Saline					
0 mg/kg	1	52.2	3	4000	0.075
Vincristine Sulfate					
0.125 mg/kg	2	53.8	6	4000	0.150
	3	45.2	38	4000	0.950
	4	42.0	25	4000	0.625
0.25 mg/kg	5	58.4	55	4000	1.375
	6	54.2	13	4000	0.325
	7	58.6	30	4000	0.750
Colchicine					
2.0 mg/kg	8	46.0	9	4000	0.225
	9	58.8	4	4000	0.100
	10	40.8	28	4000	0.700
3.0 mg/kg	11	25.6	14	4000	0.350
	12	58.0	18	4000	0.450
	13	47.4	7	4000	0.175

PCE = polychromatic erythrocyte; MNPCE = micronucleated polychromatic erythrocyte
% PCE =

Figure 3. Summary Micronucleus Data

Treatment	Number of Animals	%PCE (Mean ± SD)	Toxicity (%)	%MNPCE (Mean ± SD)	Number of MNPCE/PCE Scored
0.9% Saline					
0 mg/kg	1	52.2	---	0.08	3/4000
Vincristine Sulfate					
0.125 mg/kg	3	47.0 ± 6.1	10	0.58 ± 0.40	69/12000
0.25 mg/kg	3	57.1 ± 2.5	-9	0.82 ± 0.53	98/12000
Colchicine					
2.0 mg/kg	3	48.5 ± 9.3	7	0.34 ± 0.32	41/12000
3.0 mg/kg	3	43.7 ± 16.5	16	0.33 ± 0.14	39/12000

SD = standard deviation; PCE = polychromatic erythrocyte; MNPCE = micronucleated polychromatic erythrocyte
Toxicity = 100 – [(%PCE Test Group/%PCE Control Group)*100]

Introduction

As part of determining the mode of micronucleus formation in *in vivo* micronucleus assay, there are a couple options for identifying the nature of micronuclei available per the testing guideline (OECD 474). The most used options include anti-kinetochore (CREST; Calcinosis, Raynaud's phenomenon, Esophageal motility abnormalities, Sclerodactyly and Telangiectasia) staining and fluorescence *in situ* hybridization (FISH) with pancentromeric DNA probes. The FISH staining being used for studies with mice due to probe availability and anti-kinetochore (CREST) staining being used for studies with rats and mice. In this poster, we are investigating the use of two different aneugenic positive controls (Vincristine Sulfate and Colchicine) to determine the responsiveness for the purpose of creating a slide bank for use with anti-kinetochore (CREST) staining studies.

Methods

Male Sprague Dawley rats (8-9 weeks old) were intraperitoneally administered Vincristine sulfate at 0.125 or 0.25 mg/kg or Colchicine at 2.0 or 3.0 mg/kg. The rats were administered with the positive controls once. Bone marrow was collected 21 to 24 hours post administration. Smear slides were prepared for micronucleus evaluation prior to remaining bone marrow samples being enriched through a cellulose column for preparation of CREST micronucleus slides.

The micronucleus slides were stained with acridine orange and scored using a fluorescence microscope. Each animal had 500 total erythrocytes evaluated for the proportion of polychromatic erythrocytes (PCEs) and 4000 polychromatic erythrocytes were evaluated for the incidence of micronuclei (MNPCE).

Once the incidence of micronuclei were known, the CREST micronucleus slides for both aneugen positive controls were stained using primary antibody (anti-nuclear antibody controls, centromere (ANA)), secondary antibody (goat anti-human IgG), and propidium iodide/antifade and scored using a fluorescence microscope. At least 100 micronuclei were examined for anti-kinetochore staining for each dose group.

Results

Significant increases in the incidence of micronuclei were observed with both doses of Vincristine sulfate in the micronucleus portion of the assay (Figure 3). A greater number of micronuclei were observed in the 0.25 mg/kg dose group than in the 0.125 mg/kg dose group (Figure 2). The incidence of kinetochore positive (K+) micronuclei was greater than 65% in the CREST micronucleus assay (Table 1).

Significant increases in the incidence of micronuclei were observed with both doses of Colchicine in the micronucleus portion of the assay (Figure 3). A greater number of micronuclei were observed in the 2.0 mg/kg dose group than in the 3.0 mg/kg dose group (Figure 2). The incidence of kinetochore positive (K+) micronuclei was expected to be greater than 65% in the CREST micronucleus assay (Table 1) indicating an aneugen mode of formation. However, the results of the CREST micronucleus evaluation was low detection of the aneugenic signal. This low detection of the aneugen signal has been hypothesized to be due to improper storage conditions (*e.g.,* insufficiently thick zipper lock bag used for nitrogen purge or insufficient purge of nitrogen).

Table 1.

Treatment	Total Number of K- MN Observed	Total Number of K+ MN Observed	Percentage of K+ MN per cells scored
Vincristine Sulfate			
0.125 mg/kg	79	2	2%
0.25 mg/kg	100	2	2%
Colchicine			
2.0 mg/kg	80	3	4%
3.0 mg/kg	91	11	11%
Staining Controls (CHO-WBL cells)			
Mitomycin C, 0.1 µg/mL	85	15	15%
Vinblastine Sulfate, 7.5 ng/mL	12	88	88%

K- = kinetochore negative staining; K+ = kinetochore positive staining, MN = micronucleus
Mode of action: K+ MN ≤ 30% = Clastogen; K+ MN ≥ 65% = Aneugen; 30%< K+ MN < 65% = Mixed clastogen/aneugen

Conclusions

Both Vincristine sulfate and Colchicine induced micronuclei formation in the micronucleus assay but did not yield an aneugenic signal in the anti-kinetochore (CREST) micronucleus assay. Either positive control can be used for the creation of a positive control slide bank for micronucleus assay once a sufficiently titered dose level is obtained. Additional work is needed and is ongoing for the CREST micronucleus endpoint to determine the best storage conditions to retain the aneugen signal.

References

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OECD Guidelines for Testing of Chemicals, Guideline 474 (Genetic Toxicology: Mammalian Erythrocyte Micronucleus Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, Updated and Adopted 29 July 2016.

Schmid W. The micronucleus test. Mutation Res. 31:9-15, 1975.

Acknowledgment

CREST micronucleus slide staining procedure performed by Geoffrey Khasamba (Inotiv, Rockville).